IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	BROWN, R.S., et al.
Serial Number:	10/665,718
Filed:	22 September 2003
Title:	Detection of Biological Molecules by Differential Partitioning of Enzyme Substrates and Products
Art Unit:	1744
Examiner:	Bowers, N.A.
Confirmation No.:	4655
Attorney Docket No.:	14453

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir,

DECLARATION BY R. STEPHEN BROWN UNDER 37 C.F.R. § 1.131

I, R. Stephen Brown, hereby declare:

1. I am a professor in the Department of Environmental Studies at Queen's University at Kingston, in Kingston, Ontario, K7L 3N6, Canada.

2. I am a co-inventor on the above-captioned patent application.

3. I have reviewed the Office Action mailed on 14 December 2007. I have also reviewed the references cited in the Office Action.

4. In the Office Action, U.S. Patent Application Publication No. 2004/0047535 is one of the references cited by the Examiner in rejecting claims 24 and 54 to 57 under 35 U.S.C. § 103(a). This application was published on 11 March 2004 and filed on 9 September 2002.

5. Prior to 9 September 2002, the subject matter recited in claims 24 and 54 to 57 was invented by myself and co-inventors.

6. Exhibit "A" attached hereto is a true copy of a confidential document (15 Pages) entitled "A Method and Device for Monitoring E. coli by the Detection of Enzyme Activity in Aqueous Solution". This document bears a true date prior to 9 September 2002, and was prepared by myself and co-inventors and submitted to PARTEQ Innovations, the technology transfer office of Queen's University in Kingston, for use in preparing our U.S. Provisional Patent Application No. 60/412,015, filed on 20 September 2002, from which our above-captioned patent application claims priority.

7. Exhibit "A" describes work performed at Queen's University at Kingston, and demonstrates that the invention in claims 24 and 54 to 57 was invented and reduced to practice prior to 9 September 2002. See, for example, the first and third paragraphs of the section entitled "Our new method", the sections entitled "Methods" and "Results", and Figures 1, 3, and 4.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

R. Stephen Brown

Dec. 20, 2007
Date

Exhibit "A"

Draft - [redacted]
CONFIDENTIAL: Intended only for the Authors and officials of Parteq - Kingston.

A Method and Device for Monitoring E. coli by the Detection of Enzyme Activity in Aqueous Solution.

R. Stephen Brown, Samir P. Tabash, Eric J.-P. Marcotte, Arthur N. Ley, Ray J. Bowers, Kevin R. Hall, Moe Hussain, Peter V. Hodson and John S. Poland, Queen's University, Kingston, ON

Notes on this draft:
This is an update from the previous draft dated June 28, 2001. The previous draft included only details for detection of the enzyme β-glucuronidase, and constitutes background I.P. for the RDIS project with Precarn Inc., Hall Coastal Ltd. and Queen's University. This draft adds results for E.coli detection, with the new I.P. being covered by the RDIS project research agreement. Four author names (KRH, MH, PVH and JSP) are added as these individuals were part of the initial discussions concerning application of β-glucuronidase detection in monitoring E.coli.

Overview:
The invention described herein is a method and device for the detection of enzyme activity in aqueous solution. In this case, the enzyme is β-glucuronidase (glu), though the same scheme may be adapted to a wide variety of enzymes. The principal application which will be described is monitoring for the bacterial contaminant *Escherichia coli* (E.coli) in water, since glu is the regulatory-approved indicator of the presence of E.coli. This method may be directly applied to detection of E.coli in other samples, including meat and juice products. Future applications for this technology will include detection of other enzyme indicators of biological contaminants, measurement of enzyme activity in biological tissues such as liver, and determination of enzyme labels such as in enzyme-linked immunosorbent assay (ELISA).

Introduction:
Review of substrate methods for E. coli monitoring
The simplest methods for detection of E. coli (EC) and total coliform (TC) are based on detection of indicator enzyme activity in a broth designed to promote growth of the target organism. The indicator enzymes are β-glucuronidase (glu) and β-galactosidase (gal) for E. coli and total coliform, respectively. This approach is not necessarily foolproof, as there are potential sources of error in the suitability of broth and incubation conditions for all target coliform types, as well as in non-target organisms which may contribute to the indicator enzyme activity. Nonetheless, the reliability of established methods is high enough that there is broad regulatory acceptance of these for assessment of samples ranging from meat products to drinking water.

The methods which use indicator enzymes use reaction with a chromogenic or fluorogenic compound to measure the enzyme activity (for reviews, see ref. *1a* or *1b*). Typically, a glucuronide or galactoside conjugate of a dye compound is added to the

sample broth as a substrate, and if the target enzymes are present, then the conjugate is converted to a free dye molecule. The enzyme dependent conversion is detected by a change in colour or fluorescence of the free dye molecule compared to the conjugate. Some tests use soluble products detected in solution, with the coliform cells usually also suspended in solution. Others use the coliform cells on the surface of a filter, membrane or gel, usually with an insoluble dye product which adsorbs onto the support to form a coloured or fluorescent spot around colonies of target organisms(*5*). Some supported formats use multiple dye substrates which produce a variety of colours depending on which organisms are present.

In routine or commercial uses of these substrates, the detection is usually done visually by human eye, which presents significant limitations in performance. A large number of coliform cells must be present before enough substrate will be converted for the product to be visible. This requires significant incubation and growth for detection of a small number of initial cells, and the standard 100 ml sample is incubated for 24 hrs to provide a detection limit of one coliform cell in the initial sample. In some cases, more rapid detection is reported, but normally only with a higher detection limited accepted (e.g. 100-300 cells in a 100 mL sample)(*6,7,8*). Also, the visual detection is not quantitative, and these tests are normally used in a "presence/absence" mode where the actual number of coliform cells is not determined. The exception to the latter is some plating methods, where the number of colonies canounted and therefore cells in the sample quantitatively determined(*5*). This, however, is a very labour-intensive, time-consuming process which still requires long incubation.

A wide variety of substrates has been reported and used in commercial detection applications. For detection of conversion product in solution, fluorogenic dyes tend to be preferred. The most used example is conjugates of umbelliferone and umbelliferone derivatives (e.g. 4-methylumbelliferone, trifluoromethylumbelliferone)(*9*), favoured both because of good contrast in fluorescence between the product and the substrate, as well as rapid kinetics for reaction of conjugates with either the glu or gal enzymes. Other substrates such as dioxetane(*10*), have also been reported. Chromogenic substrates have been used in soluble detection schemes, but tend to be favoured for use in membrane or gel supported cell detection. Examples include indolyl(*11*) and indoxyl conjugates(*12*).

For our purpose, we will consider the fluorogenic conjugates producing a soluble product further here. A parallel discussion could be constructed for the other modes of detection. In choosing a substrate which will detect the lowest possible number of organisms, which requires detecting the lowest possible enzyme activity, there are three main considerations. 1) The substrate must react with the target enzyme with a high rate constant. 2) The product must be highly fluorescent such that a trace quantity is easily detected. 3) There must be a large difference in fluorescence between the substrate and the product so that the signal from the solution will change on enzyme conversion.

The third consideration above places significant limitations on the optimization of any detection scheme. Because the fluorescence change is required, this means that substrates which are used may not be the optimum in terms of rate of conversion by the

enzyme or detection limit of the product. Fluorogenic candidates which may be better substrates for the enzyme or have the most efficient fluorescent product may be ruled out if the substrate itself fluoresces, preventing detection of conversion. Many of the lowest reported detection limits for fluorophores involve polycyclic aromatic hydrocarbons, fluorescein derivatives, rhodamine derivatives, or organometallic compounds such as chelated lanthanides. None of these fluorophores is used in a glu or gal substrate because a simple derivative with the corresponding sugar has not been made which had a significantly different fluorescence spectrum or intensity compared to the product. Instead, the fluorophores used are almost all coumarin derivatives, such as umbelliferone, which have reasonable fluorescence intensity but much lower fluorescence as a conjugate with glucuronide or galactoside. As will be shown, our new approach permits the use of a much wider range of fluorophores in designing substrates.

Our new method

The working principle of the method (see Figure 1) is that a custom substrate is added to the sample solution and, upon reaction with the target enzyme, is converted to a specific product. The product is continuously monitored by a fibre-optic probe designed to produce a signal which varies linearly with the product concentration. The combination of substrate, product and fibre-optic probe is chosen such that the substrate is not detected and the product is detected at the lowest possible concentration. Specifically, in this case, the substrate is pyrenoxyglucuronide (PG), with the detected product being hydroxypyrene(HP) (Figure 2). Hydroxypyrene is measured using a fibre-optic probe which measures molecular fluorescence from a film of polydimethylsiloxane (PDMS), since HP partitions into PDMS from aqueous solution whereas PG does not partition into PDMS. The rate of appearance of HP in the PDMS film is linearly related to the concentration of enzyme in the solution, so monitoring the signal from the probe versus time provides a measurement of enzyme activity.

This novel method of detection has some distinct advantages over current methods for measuring enzyme activity. The most common solution methods use substrate/product combinations where the optical properties (absorbance of light or emission of fluorescence) change on conversion of the substrate to the product. Several substrates are available where significant optical changes do occur on enzyme conversion, but the requirement for this change places significant restrictions on the substrates which can be used. Enzymes such as glu catalyze cleavage of chemical bonds, usually by hydrolysis, which means removing an oxygen-linked substituent from the substrate and replacing it with a hydroxy group (i.e. conversion from an ether to a hydroxylated form). Conventional substrates are therefore limited to compounds which undergo a significant optical change on conversion from an ether form to a hydroxyl form. Some compounds, such as hydroxycoumarin compounds, do undergo such changes. Many other compounds which can be detected with greater sensitivity, including polycyclic aromatic hydrocarbons (PAH), do not change optical properties significantly which such a conversion.

In our new scheme, the product is distinguished from the substrate based on partition into the polymer film. This means that a change in the *chemical* properties of the compound

is required, i.e. from a form which does not partition into the polymer to a form that does, but a change in optical properties is not required. We can use a wide range of substrates not available to the conventional methods, including PAH, since they emit fluorescence both before and after conversion. In the glu example, PG is a glucuronide-form of a PAH which is fairly water-soluble and will not partition into a hydrophobic film like PDMS. The product HP is much less polar and partitions readily into PDMS.

The fibre-optic probe detection scheme offers further advantages. The entire optical path for fluorescence excitation and emission is contained inside the optical fibres and PDMS film, and light does not have to penetrate the solution in the measurement. This means that enzyme may be detected in opaque or highly scattering solutions directly. The probe provides a continuously monitored signal, which simplifies analysis of kinetics as required in enzyme activity measurements. Finally, the sensitivity of the probe is in part determined by the partition constant (K_{fs}) for the product, defined as the ratio of the compound concentration in the film to the concentration in solution. In cases where K_{fs} is greater than one, the product is 'preconcentrated' into the film. The greater the value for K_{fs}, the higher the sensitivity for detection. Values of 10,000 or greater are typical for PAHs.

Methods:

The substrate was synthesized by Ray Bowers using literature methods. While this compound has been reported in the literature, this is the first reported use for detection of β-glucuronidase activity or E.coli bacteria.

Fabrication of Optode

The optode (fibreoptic probe) consists of a single 600μm plastic-clad silica fibre (1m, Fiberguide Industries) with a polymer film affixed to one end. The optical fibre end was prepared by removing 4 cm of nylon jacket and exposing a planar surface by using a twisting and pulling technique once the fibre had been marked with a ceramic blade. The film was composed of a transparent, hydrophobic polydimethylsiloxane (PDMS) elastomer. To optimize the limit of detection, the volume of the film in the optical aperture of the fibre should be maximized, while to minimize response time, the film cross-section must be kept narrow (600μm) and/or the film must be thin (<600μm). The optodes tested were a compromise between these features. Another consideration involves shaping the film into a cone or hemisphere to limit background signal from solution, since only probe molecules in the polymer film should be detected. Three PDMS materials (different molecular weight starting materials and different degrees of cross-linking) were investigated for use as optodes.

GE RTV118 (General Electric) films were made by mixing 50 mg of the PDMS precursor material with 300 μl dichloromethane (DCM) and then allowing the DCM to evaporate until the mixture became tacky (3 hrs static or less time by blowing with nitrogen). An 18-gauge syringe tip was used to apply a bead of the tacky PDMS onto the exposed tip of the fibre and the bead allowed to cure for 24 hours at room temperature.

Sylguard 186 (Dow) films were made by mixing the base and curing agent in a 10:1 ratio by volume. This mixture was allowed to cure for 5 hours at room temperature. An 18-gauge syringe tip was used to apply a bead of mixed Sylguard 186 onto the exposed tip of the fibre and the bead allowed to cure for 48 hours at room temperature. An example of this type of bead is shown in Figure 4. The bead was then trimmed using a scalpel to remove the elastomer that is not directly in the path of the excitation light. A conical end was also fabricated using a scalpel to reduce optode volume and background scatter.

Sylguard 184 (Dow) films were made by repeating Sylguard 186 procedure, but produced much thinner films. To obtain a thicker film, the prepared fibre was placed vertically in an oven at 90°C for 30 minutes. The Sylguard 184 was prepared by mixing the base and curing agent in a 10:1 ratio by volume. While the fibre was still warm, the tip was contacted with the mixed Sylguard 184. This caused the elastomer to cure rapidly on the fibre tip and was repeated several times until the film resembles a bead of appropriate dimensions (e.g. Figure 4). The bead was then trimmed using a scalpel to remove the elastomer not directly in the path of the excitation light.

Experimental Setup

Fluorescence from the fibre probes was monitored using a fibre-coupled fluorescence spectrometer. This included a computer-controlled scanning monochromator (Sciencetech Inc., London, On.) coupled to a Xenon lamp (Sciencetech Inc., London, On.) to select excitation wavelength, and a similar monochromator with a photomultiplier detector to monitor the emission at a longer wavelength. The overall optical configuration is shown in Figure 3. This setup allowed for scanning of excitation and emission spectra, but also for monitoring emission at a fixed wavelength as a function of time, and sensor time response curves were produced.

Results:

Fibre-optic probe detection of hydroxypyrene

Initial characterization of the fibre-optic probe was done by direct addition of the product compound, 1-hydroxypyrene, to a solution containing the probe. Response with the initial probe is shown in Figure 5. Equilibration occurs in 30-40 min. for this probe, with other probes giving similar response times (data not shown). After equilibrating, the fluorescence from the probe is linearly related to sample concentration, as shown in Figure 6. A similar plot for the substrate gave a slope of zero, indicating no detection of the PG.

Fibre-optic probe detection of enzyme activity

The full operation of the probe for enzyme detection was demonstrated by inserting the probe into a substrate solution, allowing stabilization, and then adding the enzyme. After an initial delay, an increase in fluorescence was seen over 30 min (Figure 7). The slope of the curve from 20 min to 30 min was used as a measure of relative enzyme activity. The optimum substrate concentration was determined to be 10 μM by plotting measured enzyme activity at various substrate levels (Figure 8), and this concentration was used in

subsequent experiments. A plot of measured enzyme activity vs. amount of enzyme added was linear (Figure 9), indicating the ability to quantify enzyme activity with this probe, and to detect as little as 10 ng/ml enzyme levels.

Fibre-optic probe detection of E.coli

E.coli type 417 was cultured under the direction of Art Ley. Cell numbers were determined by a combination of optical density and plate count measurements. Samples containing various initial numbers of cells were placed in a glass vial (total sample 4 mL) which was thermostatted at 37.0±0.1 °C. The samples were in standard Luria broth with 10 μM substrate added. The fibre-optic probe was inserted into the sample, and fluorescence signal monitored as a function of time. After 1 to 10 hrs, the signal increased rapidly if one or more E.coli cells were initially present in the sample (see e.g. Figure 10). Control experiments (cells killed by free-thaw cycle) gave no response after 24 hr. The time between addition of the initial sample and onset of the signal (defined as an increase of 1 V above background level) was inversely related to the number of cells originally added, in agreement with a model for kinetic analysis of cell growth (see Figure 11 and following section). This demonstrates unequivocally that E.coli may be detected with the current method, substrate and instrument.

Signal versus time, kinetics of E.coli growth and quantification of E.coli

We assume that the signal versus time data obtained (see Fig. 10) may be described using a simple kinetic model for cell growth, where the fibre-optic probe is reporting the product concentration which is then a function of the number of cells present. Detailed analysis of the kinetics of product formation for each cell will follow, but is not expected to affect the model developed here. With this assumption, the probe will give a positive signal when a critical number of cells has been generated in the detection vessel. We define the initial number of cells in a sample as C_0, the number required for detection as C_d (note: cell density as number/mL can be used in place of cell number), the time for the number of cells to double as t_2, and the time required for detection as t_d. The number of cells at any time (C_t) may be written:

$$C_t = C_0 \cdot 2^{\frac{t}{t_2}}$$

and at detection time:

$$C_d = C_0 \cdot 2^{\frac{t_d}{t_2}}$$

This can be transformed to:

$$\ln(C_d) = \ln(C_0) + \frac{t_d}{t_2}\ln(2)$$

Which is rearranged to:

$$\ln(C_0) = \ln(C_d) - \frac{\ln(2)}{t_2} t_d$$

A series of experiments with known C_0 (determined separately through plate counts) were conducted, and the time to detection t_d determined. A plot of $\ln(C_0)$ versus t_d is linear (Figure 11), with the slope giving $\ln(2)/t_2$ and the intercept giving $\ln(C_d)$. From linear regression of the resulting plot, the doubling time t_2 is calculated to be 20.3 min, and the critical number of cells for detection C_d is determined to be 3×10^8.

The linearity of the plot ($R^2>0.99$) supports this model for the overall behaviour, and lets us calculate the number of cells at points in time before detection. Using this, we can now take a particular curve, e.g. C_0=1 cell, and for any specified time, we can estimate the number of cells present. When detection occurs at a specified time for an unknown sample, the same plot can be used to determine the number of cells present initially, so quantification of E.coli in the sample is possible. This assumes the same doubling time for the sample and the reference experiments, which will need to be tested for a wider variety of samples.

Future work

With detection of E.coli confirmed, the main task in the near future will be to minimize detection time. Two approaches to improving detection time are i) reduce doubling time (temperature, medium, etc.) and ii) reduce the number of cells required for generation of a signal. The current model lets us predict the impact of changes in those parameters.

Current activities include testing various media and different E.coli strains to see impact on doubling time. One sample of a second strain (E.coli K1) gave the same doubling time as indicated above. At the same time, we are testing several modifications to improve the number of cells required for detection. Modifications and expected improvement factor are: assembling a new instrument with dedicated light source and detector (10x), testing alternative substrates which can be detected with simpler/brighter light sources (10x), formulating new polymer films for the fibre sensor (10x). Depending on substrate results, we may be able to switch to a laser light source, which may provide an additional 10x detection improvement. The expected improvement of 1000 to 10000 fold will reduce the detection time for a single cell to 5-6 hrs.

Other schemes to increase the rate of substrate conversion by a particular number of cells are being considered. We expect another 10-100 fold improvement through this, which will end up with detection in 2-4 hrs.

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Figure 1: Scheme for fibre-optic probe detection of substrate conversion to product by β-Glucuronidase enzyme (glu) from E.coli cells.

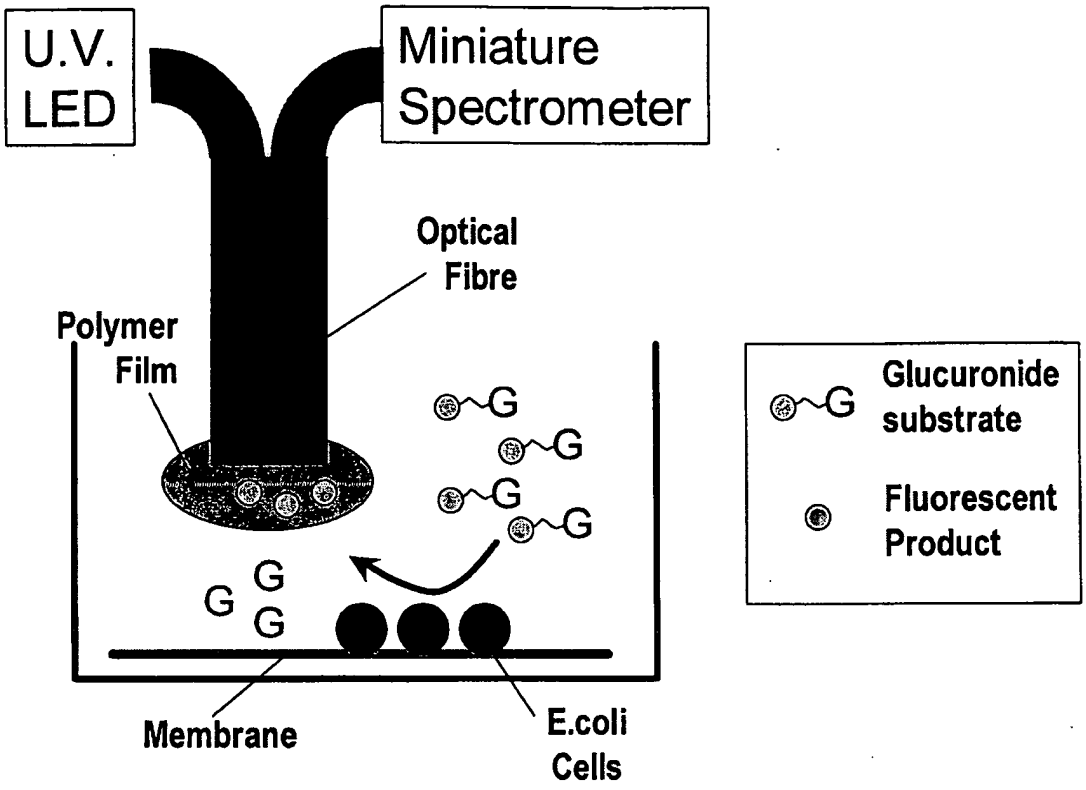
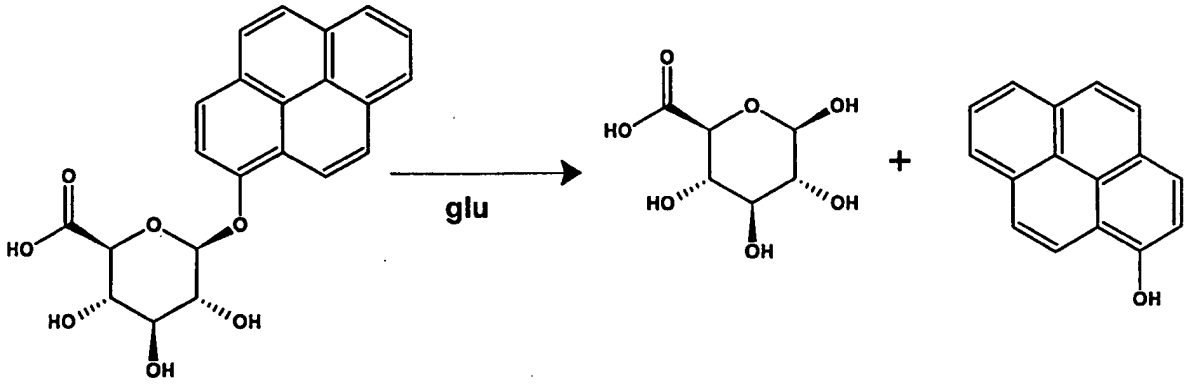


Figure 2: Reaction of pyrenoxy-β-*D*-glucuronide catalyzed by glu to produce hydroxypyrene and β-*D*-glucuronide.



Pyrenoxy-glucuronide β-*D*-glucuronide 1-hydroxypyrene

Figure 3: Sensor configuration including Probe.

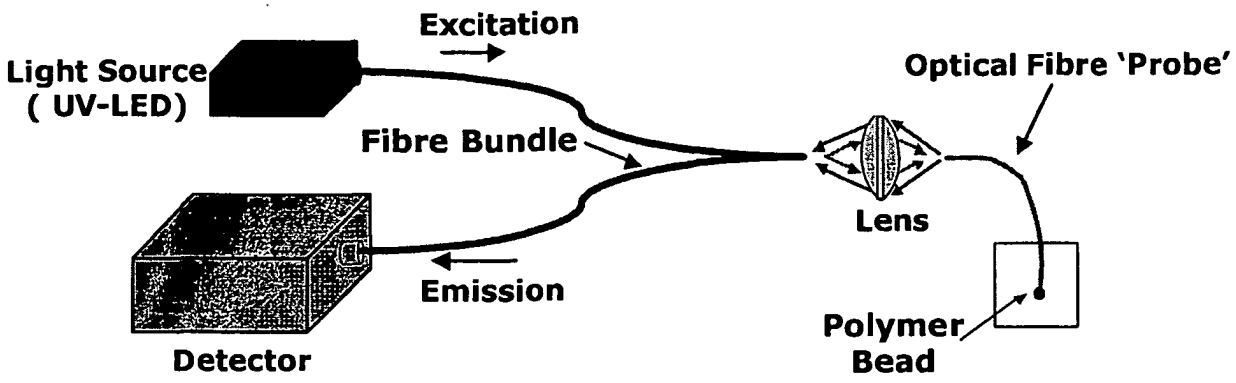


Figure 4: Polymer film on tip of 600 μm dia. optical fibre.

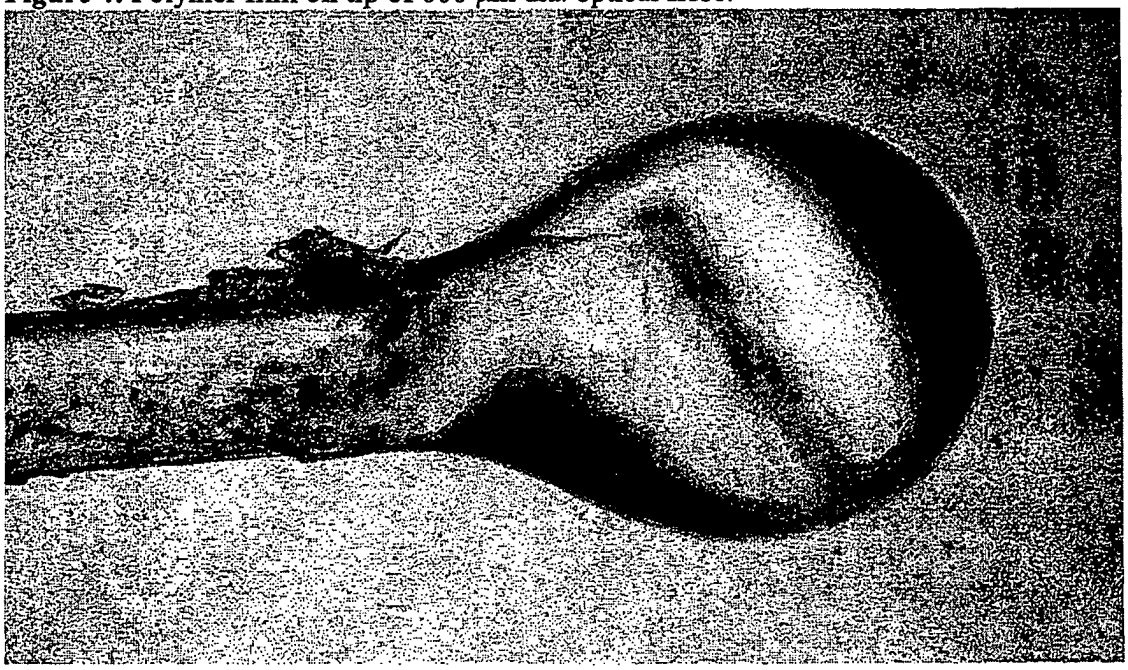
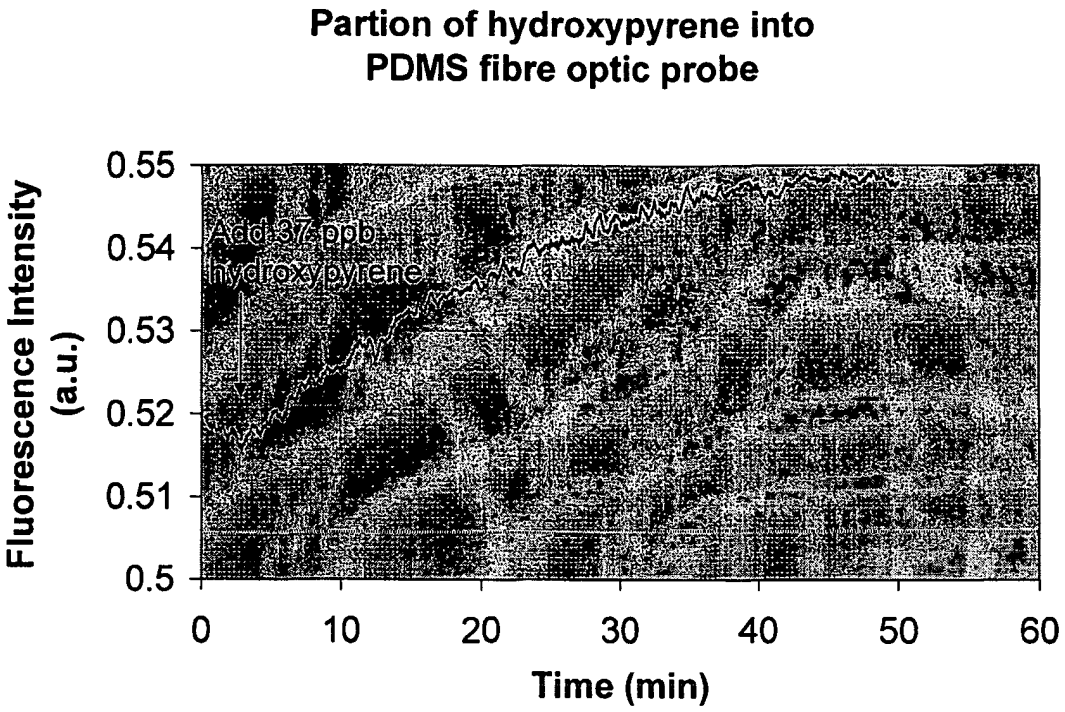


Figure 5:



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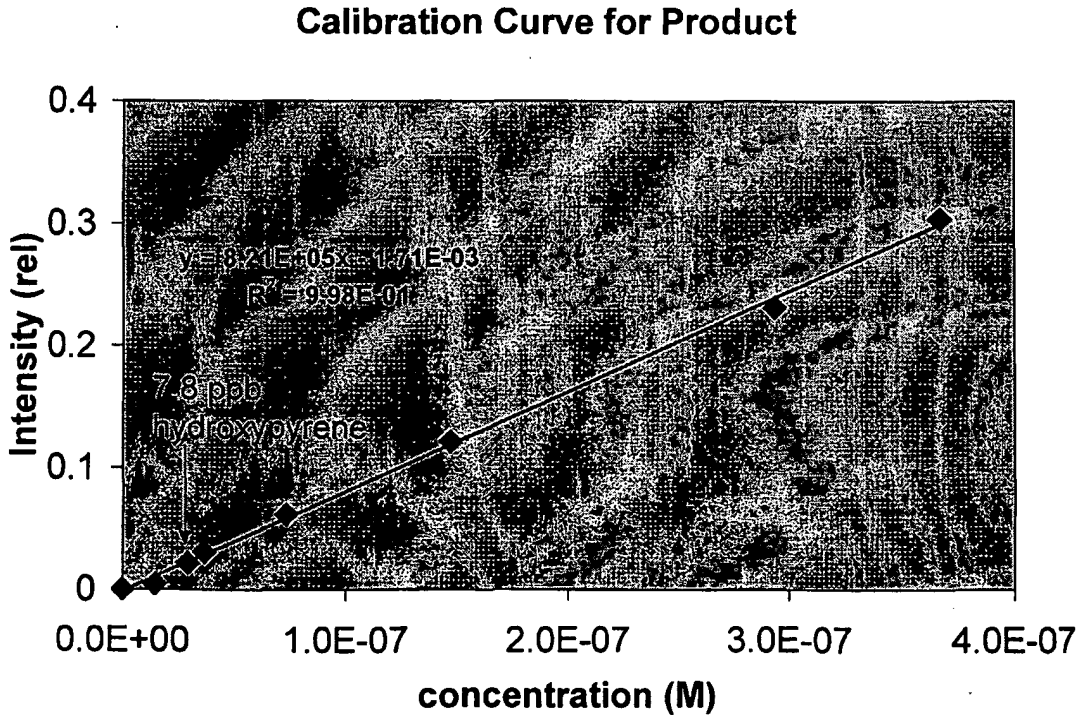


Figure 7:

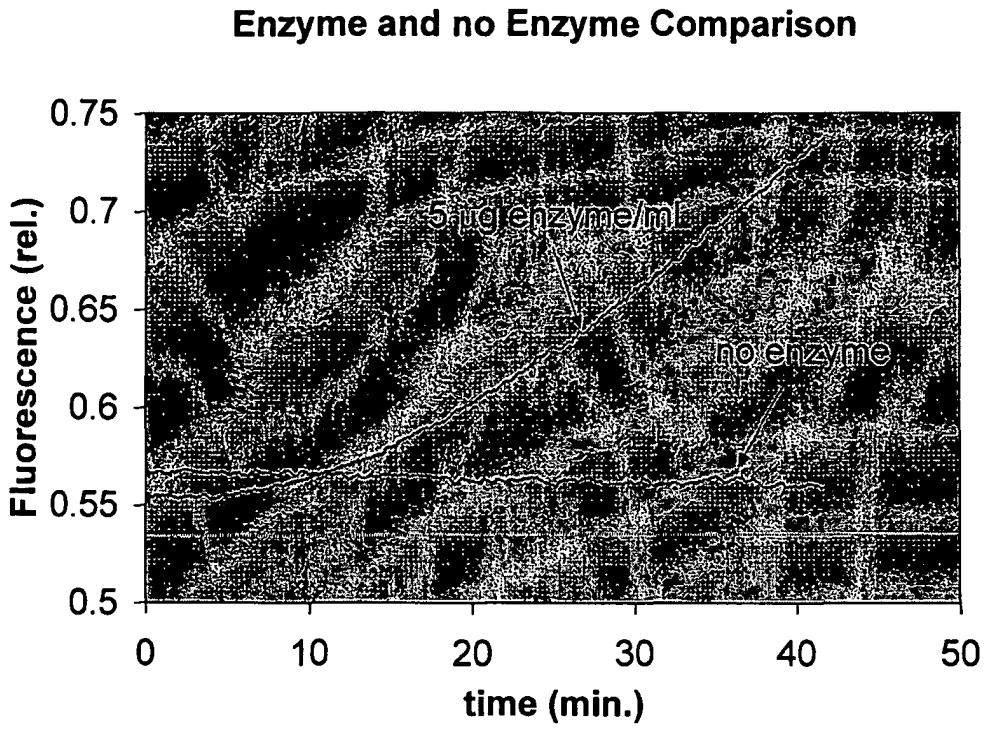


Figure 8:

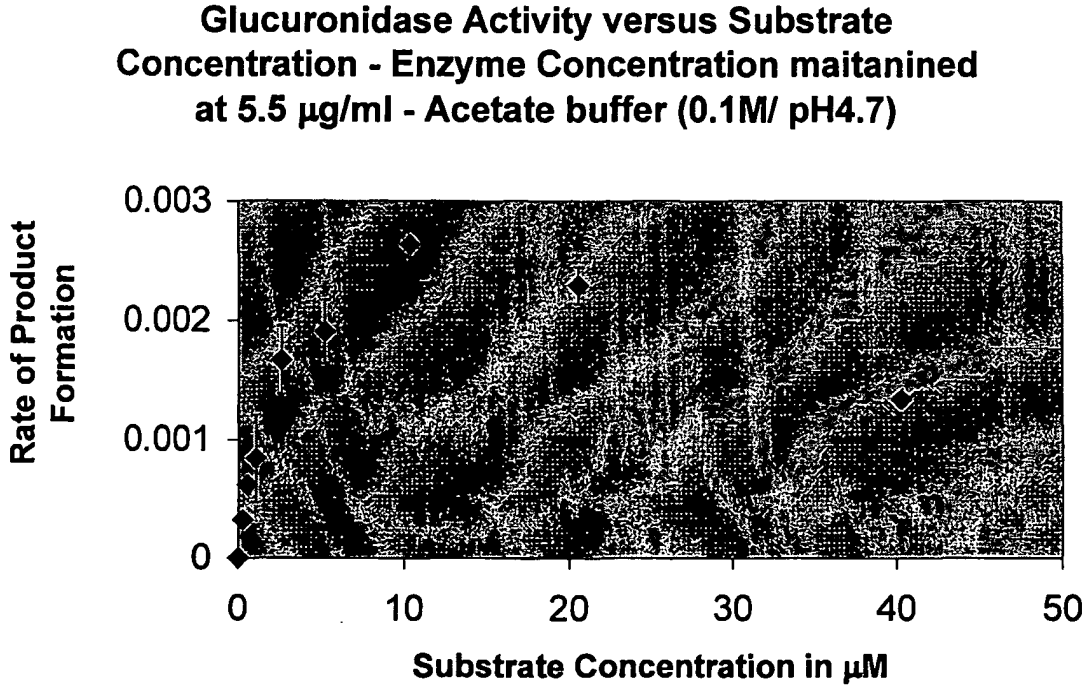


Figure 9:

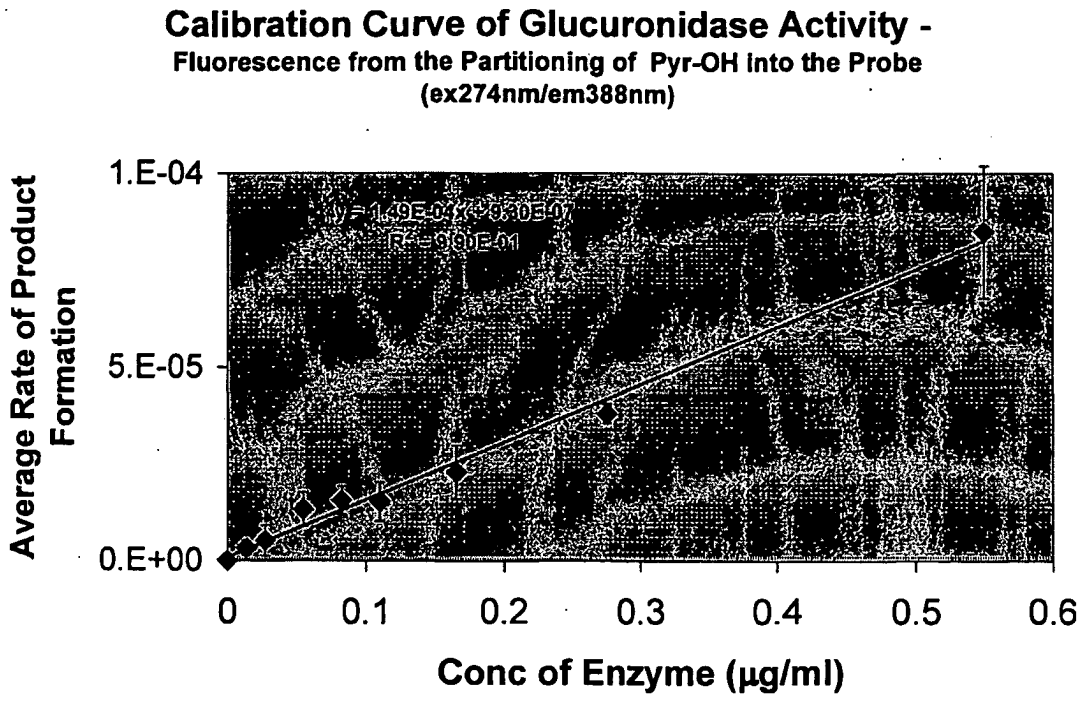


Figure 10

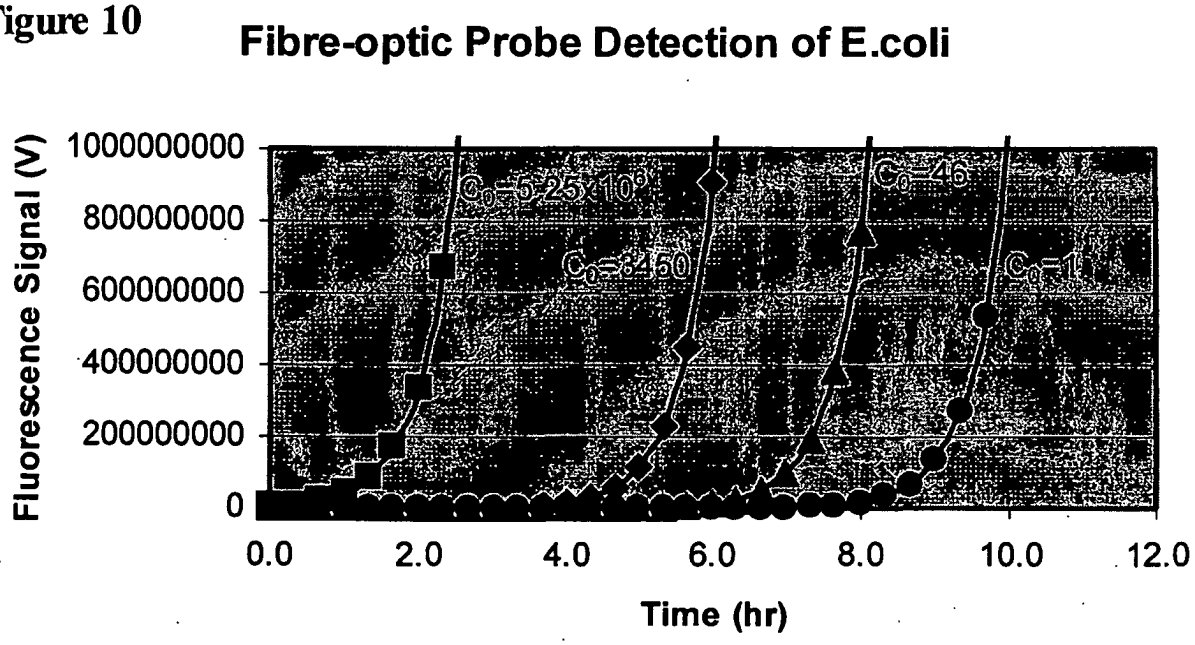
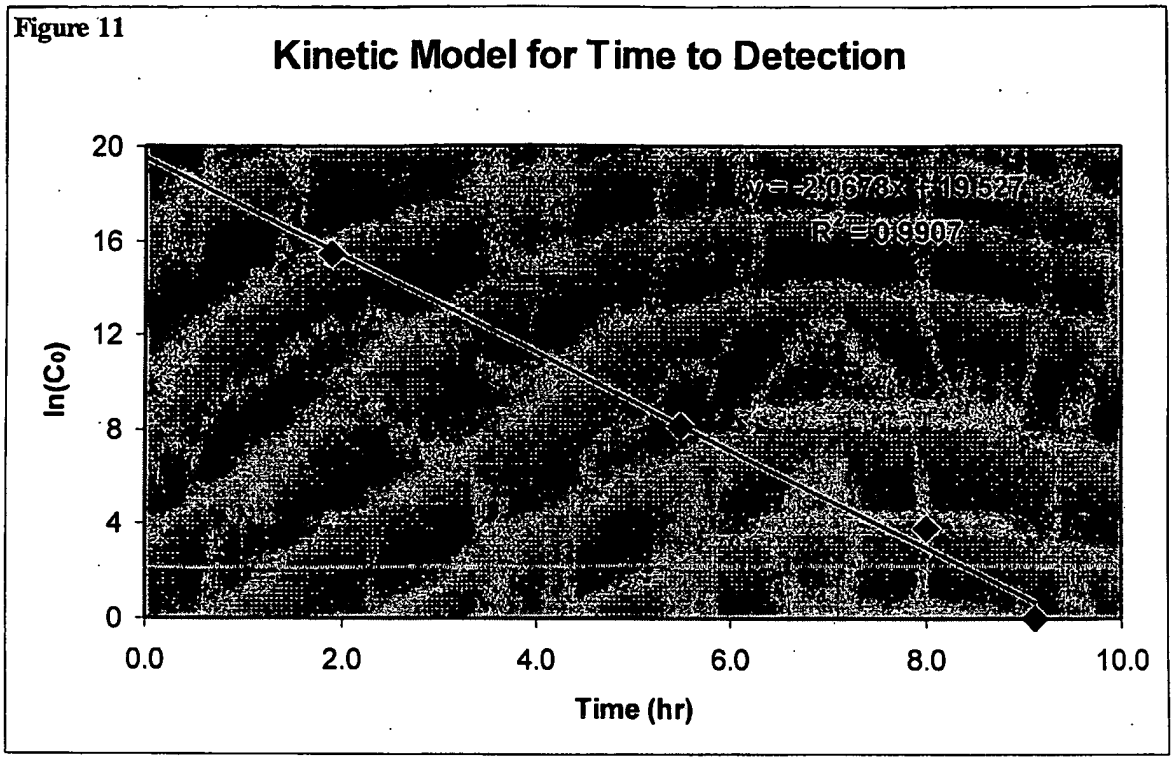


Figure 11


Kinetic Model for Time to Detection
y = -2.0678x + 19.527
R² = 0.9907
20
16
12
8
4
0
ln(C₀)
0.0
2.0
4.0
6.0
8.0
10.0
Time (hr)

I hereby certify that this correspondence of 22 pages including this certification is being transmitted by facsimile to Examiner Bowers, N.A. of the Patent and Trademark Office at Fax No. 571-273-8613 on December 21, 2007.

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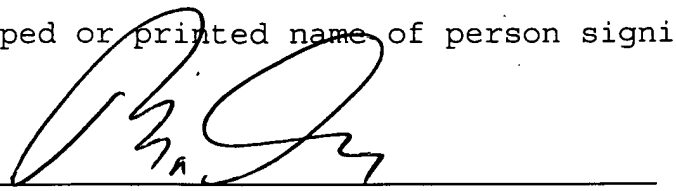

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